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Identification of Non-Muscle Nebulin Isoform in Human Brain Library

Young Mi Joo[1], Min-A Lee[1], Pyung-Rak Choi[1], Jae-Kyoung Choi[2], Yeong-Mi Lee[1], Su-Il Choi[1], Myong-Shin Kim[1], Eun-Hee Jeon[1], So-Young Kim[1] and Chong-Rak Kim[1†]

[1]Department of Biomedical Laboratory Science, and [2]Department of Biology, Inje University, Kimhae 621-749, Korea

Nebulin is a (Mr 600~900 kDa) large actin-binding protein specific to skeletal muscle and thought to act as a molecular template that regulates the length of thin filaments. Cardiac muscles of higher vertebrates have been shown earlier to lack nebulin. Recently, full-length nebulin mRNA transcripts have been detected in heart muscle, but at lower levels than in skeletal muscle. Nebulin expression also was detected in the kidney, eye, and otic canal, suggesting that nebulin isoforms may also be expressed in these organs. We have searched for nebulin isoforms in brain of human using PCR and Northern blot. Here, we provide evidence that nebulin mRNA transcripts are expressed in brain. Seven nebulin isoforms (B, C, D, E, F, G and H form) are obtained in human skeletal muscle and four isoforms (B, C, G and H form) in human brain cDNA library. We cloned the 1.3 kb of nebulin fragment from human adult brain library by PCR. The identity of the PCR product was confirmed by sequence analysis. The partial brain nebulin sequence was 99% identical to the skeletal muscle cDNA as determined by Blast alignment. It contains two simple-repeats HR1, HR2 and linker-repeats exon 135~143 except exon 140. It was different from skeletal muscle B form, which contain HR1 and HR8. These data suggest that nebulin isoform diversity occurs even more extensively than previously known, likely contributing to the distinct thin filament architecture of different striated muscles.

Key Words: Nebulin isoforms, Human brain cDNA library

INTRODUCTION

Nebulin is a filamentous protein of modular organization that comprises the fourth filament system of skeletal muscle (Mr 600~900 kDa). A single nebulin molecule associates along the entire length of the thin filament[28,30], With the C-terminus anchored in the Z-disc and the N-terminus located at the pointed ends of the thin filaments[7,18,19]. Alternative splicing in the central and C-terminal regions results in the expression of various nebulin isoforms in different skeletal muscle types, developmental stages and species, that may be altered in disease[8,14,16,19,21]. Strikingly, the molecular size of nebulin isoforms correlates with thin filament length variations in different skeletal muscle types, supporting the hypothesis that nebulin functions as a molecular template to specify the lengths of the thin filaments[11,13,28].

Sequencing studies of human nebulin cDNAs have revealed an extensively modular domain structure that appears to be ideally suited for dictating thin filament architecture [14,31]. The central region of nebulin is made up of 185 repeats that are each ~35 amino acid residues in length; these modular repeats are referred to as M1-185 and constitute 97% of the molecule[32]. Within the central region of the molecule (repeats M9 to M162), groups of seven of these modules are arranged into super repeats and share conserved SDXXYK (each repeat) and WLKGIGW (each seven repeats) motifs. Biochemical, structural, and biophysical studies suggest that a single nebulin module interacts with a single actin monomer and each nebulin super repeat interacts with a troponin-tropomyosin regulatory complex of the thin filament[5,9,12,22]. The segment comprising repeats M160-M170 links nebulin's super repeat region to the C-terminal region modules M171-M185, which are located close to the periphery of the Z-line and are characterized by a highly conserved SSVLYKEN motif. Modules M160-

*Received: January 15, 2004
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†Corresponding author: Chong-Rak Kim, Department of Biomedical Laboratory Science, Inje University, Kimhae 621-749, Korea
Tel: +82 55-320-3215, Fax: +82 55-334-3426
e-mail: bioxgeny@inje.ac.kr

M170 interact with desmin *in vitro*, suggesting that they may function in maintaining the lateral registry of adjacent myofibrils[2]. Additionally, nebulin's extreme 20 kDa C-terminus contains a serine-rich domain with potential phosphorylation sites and a src homology (SH3) domain, suggesting that nebulin is involved in signaling events within the Z-line[14]. In this regard, nebulin's SH3 domain binds to myopalladin, an interaction that appears to be critical for myofibril assembly and/or stability[3,4]. The N-terminus of nebulin contains a unique 8 kDa segment of unknown function and modules M1-M8, which contain a binding site for the thin filament pointed end capping protein, tropomodulin[18]. It has been proposed that the interaction of nebulin with tropomodulin may contribute to the regulation of thin filament lengths in muscle, an idea that supports nebulin's proposed role as a template molecule. Finally, it has been suggested that nebulin also may modulate actomyosin ATPase activity in a Ca^{2+}-dependent manner, perhaps functioning as a unique thin filament regulator[24,25].

A cardiac-specific petite nebulin, nebulette (*Mr*107 kDa), contains 22 nebulin-related modules, a C-terminal region that is virtually identical to nebulin's, and a unique N-terminal end. It binds to actin, tropomyosin, and myopalladin and is critical for myofibril assembly, thin filament organization, and contractile activity of cardiac myocytes[1,20]. Another nebulin-related protein (N-RAP), is found in intercalated discs in cardiac muscle[17]. Based on their small sizes and distribution patterns, it is unlikely that nebulette and N-RAP act as molecular templates for thin filament length specification.

Full-length nebulin mRNA transcripts have been detected in heart muscle, but at lower levels than in skeletal muscle[10]. Nebulin expression also was detected in the kidney, eye, and otic canal, suggesting that nebulin isoforms may also be expressed in these organs[20]. However it is not yet detected in brain. So we have searched for nebulin isoforms in brain using PCR and Northern blot.

Here, we provide evidence that nebulin mRNA transcripts are expressed in brain. Also four brain isoforms and seven types of skeletal isoforms are detected. These data suggest that nebulin isoform diversity occurs even more extensively than previously known, likely contributing to the distinct thin filament architecture of different striated muscles.

MATERIALS AND METHODS

1. RT-PCR

cDNA was synthesized from total RNA from human adult skeletal muscle using Superscript II (Gibco BRL). RT-PCR was performed with combination of primers (HCI: 5'-TGAGAAGTCCATGTCGTATT-3', HCII: 5'-CGTTGGGTCTCCCTCACCCG-3') designed against the human muscle nebulin sequence as described[47] using PCR system (Perkin-Elmer GeneAmp System 2400). As negative controls, PCR amplification without template added in each experiment.

2. λ phage cDNA library PCR and cloning

Human brain Large-Insert cDNA library (Clontech) were used directly as template and performed all PCRs in a volume of 50 μl containing 1X cDNA PCR reaction buffer, 10 μmol of each primer (Chong 24, λ TriplEx2 vector sequence: 5'-GAGCCCTTCGCGCGGTAACACAACCA-3' as forward primer and HCII: 5'-CGTTGGGTCTCCC-TCACCCG-3' as revers primer) and 200 μM dNTPs. PCRs were hot started by adding all reagents except DNA poly-

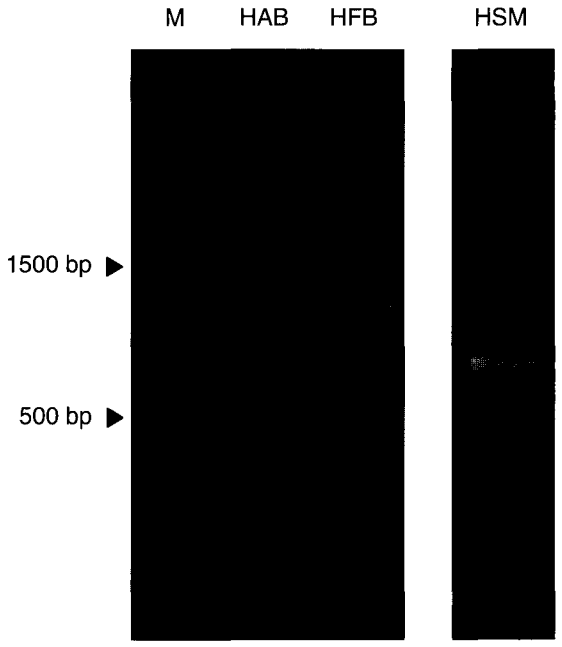

Fig. 1. PCR and RT-PCR studies on human brain library and human skeletal muscle cDNA. Four distinct isoforms are detected in human adult brain cDNA library (HAB) and human fetal brain cDNA (HFB). In human skeletal muscle cDNA (HSM) multiple size variants are detected. M, 1 kb size maker (Promega).

merase, heating to 95℃ for 10 min then holding at 80℃ for 30~60 min. Enzyme (Advantage cDNA Polymerase Mix; Clontech) was then added and amplification performed 35 cycles (95℃ for 1~2 min, 69℃ for 30 sec and 72℃ for 3 min). Amplified PCR fragment was inserted into pGEM T vector (Promega).

3. Sequencing

The coloned PCR fragments were purified QIAprep Spin Miniprep Kit (Qiagen) and sequenced with dideoxy-method using Thermo Sequenase Cycle Sequencing Kit (USB) and LI-COR 4200 (LI-COR) as described by the manufacturer. All clones were sequenced by primer walking at LI-COR. Sequence reads were compared and consensus cDNA sequences were constructed using Blast search of NCBI.

4. Northern blot analysis

Multiple Tissue Nothern Blots (Clontech) containing Human poly(A) RNA were prehybridized for 30 min in

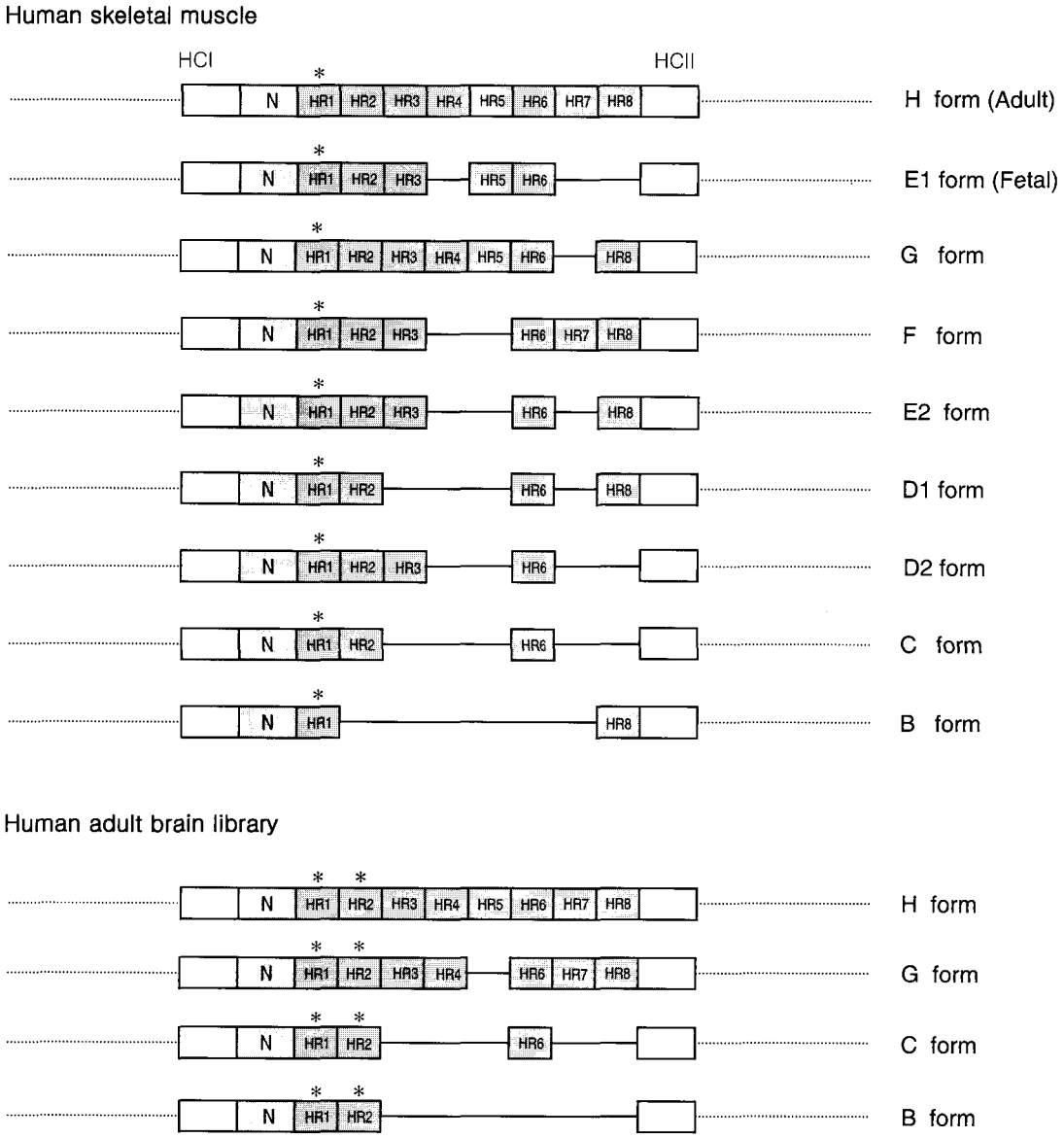

Fig. 2. Isoform diversity of the simple repeats (C termini) of human nebulin. Seven and four distinct isoforms have been observed in skeletal muscle and brain, respectively. The isoforms result from the exclusion of various combinations of the simple repeats. Thirty one-residue modules are represented HR1-HR8. Constantly expressed module is indicated with an asterisk (*).

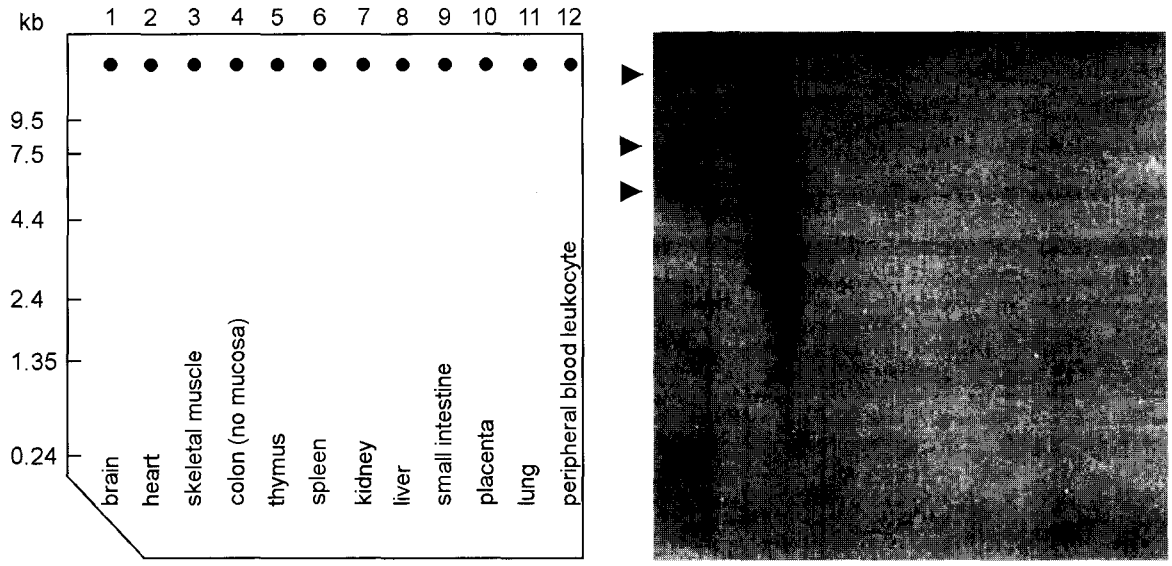

Fig. 3. Tissue distribution of nebulin mRNA. Human skeletal muscle C-form of nebulin cDNA probe was hybridized to human MTN Blot (Clontech). Nebulin transcripts were detected in skeletal muscle and brain. The abundant transcripts were detected in skeletal muscle more than brain.

ExpressHyb Solution (Clontech) at 68℃. Then the membrane was hybridized for 1 h at 68℃ with [^{32}P]dCTP-labeled skeletal muscle C-type cDNA probe. Autoradiography was performed at -70℃ for 24~48 h with intensifying screen.

RESULTS

RT-PCR and Library PCR studies using HCI-sense and HCII-reverse primer pairs showed that nebulin isoforms are expressed in human brain and skeletal muscle (Fig. 1). We found that the majority of nebulin PCR products were detectable in both brain and skeletal muscle cDNAs (Fig. 1). Seven nebulin isoforms (B, C, D, E, F, G and H form) are obtained in human skeletal muscle and four isoforms (B, C, G, H form) in human adult and fetal brain cDNA library (Fig. 2). However, using semi-quantitative PCR analyses with ethidium bromide staining, we found that an additional five to eight cycles were required to amplify the products from brain cDNA compared to skeletal muscle cDNA (data not shown). These data suggest that while nebulin mRNA transcripts are expressed in brain, they are not as abundant as nebulin transcripts expressed in skeletal muscle; this may explain why they were weakly detected in brain by Northern blot analysis (Fig. 3).

We cloned the 1.3 kb of nebulin fragment from human

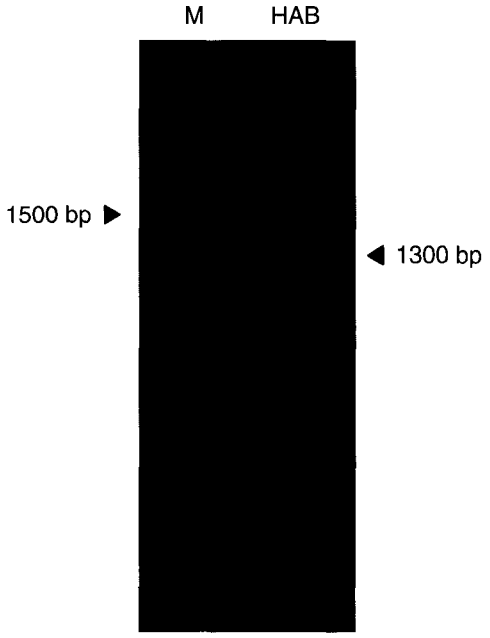

Fig. 4. Cloning PCR product of human adult brain library. PCR product of 1.3 kb of nebulin fragment was obtained by using vector specific primer (Chong 24) and HCII. M, 1 kb size maker; HAB, PCR product of human adult brain library cDNA. The product was inserted into pGEM-T vector.

adult brain library by PCR using primers Chong 24 and HCII (Fig. 4). The identity of the PCR product was confirmed by sequencing. The partial brain nebulin sequence was 99% identical to the skeletal muscle cDNA as deter-

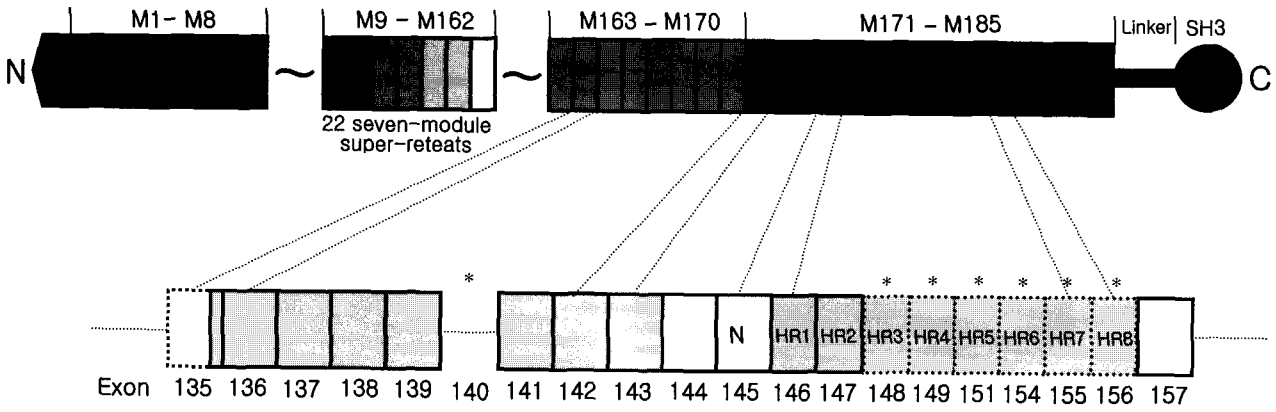

Fig. 5. Comparison of the muscle nebulin (top) with the brain nebulin (below). The modules and the exons of nebulin were aligned. Exons 135~144 encode the linker repeats and exons 146-156 encode the simple repeats. Exon 140 and exons 148~156 (HR3~HR8) that are differentially expressed in striated muscle and brain indicated with an asterisk (*). Exon 150 and exons 152~153 are spliced.

mined by Blast alignment. It contains two simple-repeats HR1, HR2 and linker-repeats exon 135~143 except exon 140 (Fig. 5). It was difference from skeletal muscle B form, which contain HR1 and HR8 (Fig. 2).

We perfomed the alignment of the brain nebulin gene sequence to the complete human[14] (accession code X-83957) skeletal muscle nebulin cDNA sequences. Exons 135~143 are located in the linker-repeat region (Fig. 5) and exons 146~156 are encode additional nebulin simple repeat modules with a SSLVYKEN consensus sequence. Exon 150 and exones 152~153 are spliced (Fig. 5). Differential expression of exons 148~156 may therefore contribute to Z-line architecture.

DISCUSSION

Here, we report that the expression of nebulin mRNA transcripts in brain has been detected by PCR and Northern blot studies. These data suggest that while nebulin mRNA transcripts are expressed in brain, they are not as abundant as nebulin transcripts expressed in skeletal muscle. This may explain why they were not detected in heart[14,33,26] and weakly detected in brain by Northern blot analysis. Interestingly, a primer pair from nebulin's Z-line region amplified multiple isoforms from different exon skipping events in the exon segment 147~160 in human skeletal muscle, the human brain nebulin isoform composition appeared to be different; seven fragments were amplified from skeletal muscle cDNA, whereas only four fragments were amplified from brain cDNA (Fig. 1, 2). This difference may reflect variations in actin-binding properties between skeletal muscle and brain.

The skeletal nebulin C-terminal SH3 domain interacts with myopalladin[3], regions of nebulin that are present in brain. The skeletal muscle nebulin domains M160~M176, previously shown to contain a binding site for the intermediate filament protein, desmin[2], are included in the brain nebulin isoform. The conservation of these ligand-binding sites in brain and skeletal nebulins suggest that nebulins may have conserved roles in brain and skeletal muscle.

In summary, our data now suggest that the extent of nebulin splice isoform diversity in muscle is greater than previously known, and likely includes the expression of specific nebulin isoforms in brain. We speculate that many of nebulin's proposed roles, including that of a thin filament ruler, are similar in skeletal and cardiac tissue. However, future investigations are required to investigate how they may work together with proteins, such as tropomodulin, to regulate thin filament lengths. It is tempting to speculate that multiple isoforms of nebulin are co-expressed in other muscles, which may correlate with the variations in thin filament lengths observed in individual myofibrils[23,29]. In this regard, smaller and larger nebulin isoform co-expression may be analogous to the co-expression of various titin isoforms, which function independently[6,8,12].

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